MICROENCAPSULATED NITRIC OXIDE SYNTHASE SOURCE

Field of the Invention

This invention relates to microencapsulated recombinant cells for *in vivo* delivery of therapeutic substances.

Background to the Invention

Experiments have demonstrated that nitric oxide (NO) can have beneficial effects in a number of disease states. However, NO can be a double edged sword: the high levels of NO produced by macrophages and endothelial cells can have a tumor killing effect, whereas low and medium doses of NO may actually promote tumor growth, upregulation of p53 and tumor angiogenesis. Control of NO levels *in vivo*, however, is difficult to achieve.

A well established pharmacological NO-based therapeutic approach is to add NO in some form. One way to do this is simply to add authentic NO solution. However, this approach is problematical, as pure NO solution is very unstable in aqueous media.

An alternative and widely used approach is to use NO-donors, which can release NO continuously in a biological environment. Compounds such as glycerin trinitrate (GTN), sodium nitroprusside (SNP) and S-nitroso-N-acetyl-DL-penicillamine (SNAP) are widely used therapeutically. However, a problem with all classical NO donors is that NO release is often not linear. Furthermore NO-donors may also give rise to related NO species that have different biological properties from native NO. For example, NO may react with oxygen derived radicals to produce toxic substances such as the peroxynitrite anion (ONOO-).

Recent attempts to overcome the problems associated with NO donors have focussed on the use of targeted gene delivery approaches. For example, vessel walls transfected with an iNOS cDNA following angioplasty show reduced restenosis. This method is, however, limited in that it may be difficult to regulate expression of the iNOS from such cells and it is difficult to transfect all the cells in a specific target region.

Summary of the Invention

We have produced human cell lines that can express one of the three known human nitric oxide synthases, inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS), in the presence of the insect steroid hormone ecdysone or an analog thereof. We have also produced a cell line that can express human iNOS in the presence of tetracycline or an analog thereof.

Thus, we have produced cell lines which are capable of producing NO in a dose- and time-dependent manner by treatment of the cells with ecdysone or an analog thereof or tetracycline or an analog thereof. Our cell-based NO "switches" produce biologically active inducible NOS capable of generating NO.

Furthermore, we have encapsulated a cell line, which expresses iNOS under the control of an ecdysone-responsive promoter, in immunoisolating microcapsules. We have shown that under *in vitro* culture conditions, the cells can survive for longer than one month. Furthermore, the encapsulated cells could be induced to produce NO at a controlled level upon stimulation by muristerone A. When muristerone A was removed, the NO levels fell. Cells could be "switched on" again by the reapplication if muristerone A.

When microencapsulated cells of the invention were implanted into mice, they survived and upon removal from the host mice were still capable of induction by muristerone A and of NO production.

We have now shown, in a nude mouse xenograft model that the microencapsulated cells have the ability to slow down tumor growth and even to completely inhibit tumor growth. This remarkable result opens up a completely new strategy for cancer therapy.

Thus, microencapsulated cells of the invention may be used as *artificial endothelial cells* or as *artificial macrophage cells* in the treatment of human conditions related to abnormal NO metabolism or conditions where the administration of nitric oxide is required. This approach has advantages over NO-donor and gene therapy approaches as 100% of the cells introduced into a target region will be capable of generating NO in a highly regulated way.

According to the present invention there is thus provided microcapsules suitable for administration to a human or animal which microcapsules harbour cells containing a polynucleotide construct, said construct comprising:

(a) a promoter which is responsive to ecdysone or an analog thereof and which is operably linked to a coding sequence for a nitric oxide synthase (NOS) or a functional variant thereof; or

(b) a promoter operably linked to one or more tetracycline operator site sequences and a coding sequence in that order, wherein the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof.

The invention also provides:

- microcapsules of the invention for use in a method of treatment of the human or animal body;
- use of microcapsules of the invention for the manufacture of a medicament for use in the treatment of a condition associated with deficient NO production;
- a pharmaceutical composition comprising microcapsules of the invention and a pharmaceutically acceptable carrier or diluent;
- a method of delivering microcapsules to a host comprising administering microcapsules of the invention to the host;
- a method of treating a host suffering from a condition associated with deficient NO production, which method comprises administering ecdysone or an analog thereof to a host which harbours microcapsules of the invention.
- a product containing microcapsules of the invention and ecdysone or an analog thereof as a combined preparation for simultaneous, separate or sequential use in the treatment of condition associated with deficient NO production;
- a polynucleotide construct comprising:

(a) a promoter operably linked to a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the

coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof; or

(b) a promoter operably linked to one or more tetracycline operator site sequences and a coding sequence in that order, wherein the coding sequence encodes a nitric oxide synthase (NOS) or a functional variant thereof;

- a vector which incorporates a polynucleotide construct of the invention;
- a process for preparing microcapsules comprising encapsulating cells of the invention.

Brief description of the Drawings

Figure 1(a) shows the plasmid map for pIND-hiNOS-f (human iNOS); Figure 1(b) shows the plasmid map for pIND-hnNOS-f (human nNOS); and Figure 1 (c) shows the plasmid map for p-IND-heNOS-f (human eNOS). Figure 1(d) shows the plasmid map of pTet-hiNOS-f (human iNOS).

Figure 2(a) shows generation of NO by EcR293 clone 11, following treatment with muristerone A. EcR293 clone 11 cells were grown with varying concentrations of muristerone A and at different time intervals supernatants were taken and the Griess reaction was used to measure the nitrite concentration. Figure 2(b) shows Northern and Western blots of carried out on extracts from cells treated with either 1μM or 10μM muristerone A. Extracts were also taken from cells grown in the absence of muristerone A. For Northern blots, filters were probed with a human iNOS cDNA and hybridisation with human β-actin was used as a loading control. For Western blots, filters were probed with a polyclonal antibody raised against the 7 C-terminal residues of human iNOS: Cyc-Arg-Nle-Orn- (Ser-Leu-Glu-Met-Ser-Ala-Leu). The filters were subsequently stripped and re-probed with an anti-human α-tubulin antibody as a loading control.

Figure 3 shows generation of NO in a panel of cell lines transfected with pTet-hiNOS-f. Cells were treated with 1μg/ml tetracycline for 24h and NOS activity

was assessed by assaying for accumulated nitrite using the Griess reaction.

Figure 4 shows the general strategy used for microencapsulation of cells. In step 1, EcR293 clone 11 cells are mixed with Na^+alginate in the barrel of a syringe. In step 2, the resulting solution is expelled dropwise through the syringe needle into a solution of $CaCl_2$ giving rise to polymer beads containing EcR293 clone 11 cells. In step 3, the microencapsuled are hardened by mixing with poly-L-lysine. In step 4, the microcapsules are ready for *in vitro* culture or *in vivo* injection/transplantation. Alternatively, microcapsules may be stored in DMEM medium supplemented with 10% fetal bovine serum.

Figure 5 shows generation of NO by encapsulated EcR293 clone 11 cells, following treatment with muristerone A. EcR293 clone 11 cells were encapsulated with alginate-poly-L-lysine-alginate. Cells were induced to produce NO at a controlled level by the application of 10μM muristerone A. When muristerone A was removed the production of NO fell. On re-application of 10μM muristerone A the level of NO production again increased.

Figure 6 shows the results of experiments carried out with microencapsulated EcR293 clone 11 cells. (A) shows the results of subcutaneous injection of human colon cancer DLD-1 cells ($1x10^6$ per mouse) with microencapsulated human EcR293 clone 11 cells ($5x10^6$ per mouse) either under induction conditions (ponasterone A 1mg per mouse, ▫) or sesame-seed oil only (◆). Each point represents mean±S.D. for 5 mice.

Detailed Description of the Invention

Constructs, Vectors and Cells

Steroid hormones are small hydrophobic molecules that can diffuse through the plasma membrane of cells where they can bind reversibly to specific steroid-hormone-receptor proteins in the cytoplasm or nucleus. The binding of hormone

activates the receptor, enabling it to bind with high affinity to specific DNA sequences that act as transcriptional enhancers. This binding increases the level of transcription from certain nearby genes.

A pulse of the insect steroid hormone ecdysone triggers metamorphosis in *Drosophila melanogaster*, showing effects such as chromosomal puffing within minutes of hormone addition. Mediating this response is the functional ecdysone receptor, which is a heterodimer of the ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP).

Insect hormone responsiveness can be recreated in cultured mammalian cells by cotransfection of a cell with a functional ecdysone receptor (a heterodimer of EcR and USP) and an ecdysone responsive construct and treatment of the cell with ecdysone or an analog thereof.

A tetracycline responsive system can be created in cultured mammalian cells by cotransfection of a cell with a plasmid encoding a tetracycline repressor protein (tetR) and a plasmid containing a tetracycline responsive element linked to a promoter. The promoter sequence is used to drive heterologous gene expression. The tetracycline responsive element comprises particular DNA sequences called tetracycline operator sites, which can bind a homodimer of tetR. If those sequences are positioned between a promoter and a coding sequence in a construct, the presence of tetR bound to a tetracycline operator site will prevent the promoter driving expression of the coding sequence. However, when tetracycline is added to cells the tetracycline binds to tetR homodimers leading to a conformational change in tetR, such that it is unable to bind a tetracycline operator site. The tetR:tetracycline complex dissociates from the Tet operator site and allows the promoter to drive expression of the coding sequence.

(i) Constructs

The invention provides polynucleotide constructs which are responsive to ecdysone or an analog thereof. The invention also provides polynucleotide constructs which are responsive to tetracycline or an analog thereof.

The ecdysonse responsive constructs comprise a promoter operably linked to

a coding sequence, wherein the promoter is responsive to ecdysone or an analog thereof and the coding sequence codes for a NOS or a functional variant or fragment thereof.

The tetracycline responsive constructs comprise a promoter operably linked to one or more teracycline operator site sequences and a coding sequence in that order, wherein the coding sequence codes for a NOS or a functional variant or fragment thereof.

The constructs may comprise DNA or RNA. They may also include within them synthetic or modified nucleotides. A number of different types of modification to polynucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the invention, it is to be understood that the constructs described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or lifespan of constructs of the invention. Constructs of the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. Thus, a regulatory sequence, such as a promoter, "operably linked" to a coding sequence is positioned in such a way that expression of the coding sequence is achieved under conditions compatible with the regulatory sequence.

A promoter for use in an ecdysone-reponsive construct of the invention may be any promoter which can drive the transcription of a coding sequence to which it is operably linked in the presence of the steroid hormone ecdysone or an analog thereof.

The promoter may be a naturally occurring promoter from a *Drosophila melanogaster* or other insect ecdysone-responsive gene. Alternatively, the promoter may be a non-naturally occuring promoter. A non-naturally occuring promoter may be used which comprises a minimal promoter and an ecdysone-responsive element (EcRE). An EcRE is a nucleotide sequence to which a functional ecdysone receptor can bind in the presence of ecdysone. Suitable minimal promoters include the minimal heat shock promoter.

An ecdysone-responsive promoter may comprise more than one EcRE, for example 2 to10 elements or more preferably 4 to 6 elements. The sequence of an EcRE will depend on the exact functional ecdysone receptor used. If a modified functional ecdysone receptor is used (see below) it may be appropriate to use a modified EcRE (see No *et al.*, *Proc. Natl. Acad. Sci. USA*, **93**: 3346-3351). The EcRE(s) and minimal promoter sequences do not have to be immediately adjacent. Because EcREs function as transcriptional enhancers, they can be placed some distance upstream, for example from 1, 10 or 25 nucleotides to 30, 40, 50, 100, 500 or 1000kb upstream of a minimal promoter. EcREs could even be placed further than 1kb upstream of a minimal promoter. Generally, if multiple copes of an EcRE are used, the mutiple copies will be arranged in an array, one after the other.

Constructs of the invention may be responsive to ecdysone [(*2β, 3β, 5β, 22R*)-2.3,14,22,25-pentahydroxycholest-7-en-6-one] or an analog thereof. Suitable analogs of ecdysone for use in the invention include muristerone A [2β, 3β ,5α, 11α, 14, 20, 22-heptahydroxy-5β, 7-cholesten-6-one] or ponasterone A [(*2β, 3β, 5β, 22R*)-2, 3, 14, 20, 22, 25-pentahydroxycholest-7-en-6-one] and GS™-E (Invitrogen, San Diego, CA; see also Dhadialla *et al.*, 1998, Ann. Rev. Entomol. **43**: 545-569).

A promoter for use in an tetracycline-reponsive construct of the invention may be any promoter which can drive the transcription of a coding sequence to which it is operably linked in the presence of the antibiotic tetracycline or an analog thereof.

Generally, the choice of promoter will depend on the host cell to be used for expression of the coding sequence. Typically, expression in mammalian cells, for example human cells will be required and thus a mammlian promoter will be preferred. Mammalian promoters, such as β-actin promoters, may be used. Tissue-specific promoters may be used. Viral promoters may also be used, for example the Moloney murine leukaemia virus long terminal repeat (MMLV LTR), the rous sarcoma virus (RSV) LTR promoter, the SV40 promoter, the human cytomegalovirus (CMV) IE promoter, adenovirus, HSV promoters (such as the HSV IE promoters), or HPV promoters, particularly the HPV upstream regulatory region (URR). Viral promoters are readily available in the art. Constitutive promoters, for example the

CMV promoter, are preferred.

Tetracycline-responsive constructs comprise one or more tetracycline operator site ($TetO_2$) sequences, situated between the promoter and coding sequence. For example, two, three, four or even up to ten $TetO_2$ sequences may be used. Typically, if more than one $TetO_2$ site is used those sites will be arranged in the form of an array. However, other intervening nucleotide sequences may be situated between individual $TetO_2$ sites. For example one, two, three, four, five, up to ten or up to 15 nucleotides may intervene between any two $TetO_2$ sites.

The $TetO_2$ sequence is 5'-TCCCTATCAGTGATAGAGA-3' (Hillen and Berens, 1994, Annu. Rev. Microbiol. **48**, 345-369; Hillen *et al.*, 1983, J. Mol. Biol. **169**, 707-721) or a functional variant thereof. The $TetO_2$ sequence or a functional variant thereof is capable of being bound by a homodimer of tetR or a functional variant thereof.

A functional variant of the $TetO_2$ sequence is a sequence which is similar to that of the $TetO_2$ sequence and which remains capable of binding a homodimer of tetR or a functional variant thereof. The affinity of tetR for the $TetO_2$ sequence is $K_B = 2x10^{11}\ M^{-1}$ (as measured under physiological conditions), where K_B is the binding constant (Hillen and Berens, 1994, *supra*). The binding affinity of tetR for a functional variant of the $TetO_2$ sequence may be substantially the same as that of tetR for the $TetO_2$ sequence. Alternatively, tetR may have a binding affinity for a functional variant of the $TetO_2$ sequence which is greater or less than that of tetR for the $TetO_2$ sequence. For example, the affinity of tetR for a functional variant of the $TetO_2$ sequence may be from $K_B = 2x10^{9}\ M^{-1}$ to $2x10^{13}\ M^{-1}$ or more preferably from $2x10^{11}\ M^{-1}$ to $2x10^{12}\ M^{-1}$.

A functional variant of $TetO_2$ typically comprises a sequence substantially similar to that of the $TetO_2$ sequence. Thus, a functional variant of $TetO_2$ will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to the $TetO_2$ sequence, calculated over the full length of those sequences.

A functional variant of the $TetO_2$ sequence may be a modified version of that sequence obtained by, for example, nucleotide substitution or deletion. Up to 1, up

to 2, up to 3, up to 4, up to 5, up to 6 or more nucleotide substitutions or deletions or combinations thereof may be made to the $TetO_2$ sequence to produce a functional variant of that sequence.

Constructs of the invention may be responsive to the antibiotic tetracycline or an analog thereof. Tetracycline binds to tetR homodimers, such that the tetR:tetracylcine complex dissociates from the $TetO_2$ sequence. The association constant of tetracycline to tetR is $3 \times 10^9\ M^{-1}$. Preferred analogs of tetracycline will have an association constant substantially similar to or greater than that of tetracycline for tetR. Suitable analogs of tetracycline include doxycycline. Doxycycline exhibits similar dose response and induction characteristics with constructs of the invention, but has a longer half-life than tetracycline (48 hours vs. 24 hours respectively).

The coding sequence used in both ecdysone- and tetracycline-responsive contructs of the invention can be any sequence which encodes a NOS or a functional variant thereof. The phrase "nitric oxide synthase" is intended to include all naturally occuring forms of iNOS, nNOS and eNOS as well as variants which retain NOS activity, for example variants produced by mutagenesis techniques. Preferably the coding sequence encodes a NOS of mammlian origin for example rodent (including rat and mouse) or human. Most preferably the coding sequence encode the human iNOS (GenBank accession number: X73029, Coding sequence 226-3687), human nNOS (GenBank accession number: U17327, Coding sequence 686-4990) or human eNOS (GenBank accession number: M95296, Coding sequence 21-3632) or a functional variant of any one of those enzymes.

A functional variant of a NOS is any polypeptide which demonstrates NOS activity, for example a fragment of a NOS. A coding sequence which codes for a functional variant of a NOS may be, for example a fragment of a full length NOS coding sequence. A fragment may be of any length, so long as the polypeptide for which it codes has NOS activity.

A functional variant of a NOS typically comprises a sequence substantially similar to that of the naturally occurring form of the relevant NOS sequence. Thus, a functional variant of a NOS will generally have at least 60%, at least 70%, at least

80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to the relevant NOS sequence, calculated over the full length of those sequences.

Thus, the coding sequence may be modified by nucleotide substitutions or deletions. For example up to 1, 2 or 3 to 10, 25, 50, 75 or 100 substitutions or deletions or combinations thereof may be made to produce a functional variant of a NOS. A polynucleotide encoding a NOS may alternatively or additionally be modified by one or more insertions and/or deletions and/or by an extension at either or both ends. The modified polynucleotide generally encodes for a polypeptide which has NOS activity. Degenerate substitutions may be made and/or substitutions may be made which would result in a conservative amino acid substitution when the modified sequence is translated, for example as shown in the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other.

ALIPHATIC	Non-polar	G A P
		I L V
	Polar-uncharged	C S T M
		N Q
	Polar-charged	D E
		K R
AROMATIC		H F W Y

Sequence identity may be calculated as follows. The UWGCG Package provides the BESTFIT program which can be used to calculate identity (for example used on its default settings) (Devereux *et al* (1984) *Nucleic Acids Research* **12**, p387-395). The PILEUP and BLAST algorithms can be used to calculate identity or line up sequences (typically on their default settings), for example as described in Altschul S. F. (1993) J Mol Evol 36:290-300; Altschul, S, F *et al* (1990) J Mol Biol 215:403-10. Software for performing BLAST analyses is publicly available through the National Centre for Biotechnology Information (**http://www.ncbi.nlm.nih.g v/**).

(ii) Vectors

Both types of construct of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the construct in a compatible host cell. A vector may also provide for expression of the NOS coding sequence when the vector is harboured by an appropriate host cell. The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication and optionally a regulator of the ecdysone-responsive promoter or promoter used in a tetracycline-responsive construct.

The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene for selection in bacterial cells or a G418 or a zeocin resistance gene for selection in mammalian cells.

Vectors of the invention may also comprise so-called "suicide genes", for example the herpes simplex virus thymidine kinase gene (HSV-tk). The HSK-tk gene may be induced in the presence of a small molecule such as ganciclovir, resulting in the elimination of a cell harbouring the HSV-tk gene. Alternatively, a cell which harbours a vector of the invention which does not comprises the HSK-tk gene may be provided with the HSV-tk gene by integration of the HSV gene into the host cell genome or by transfection or transformation of the cell with a further vector comprising the HSV-tk gene.

(iii) Cells

Vectors of the invention, which incorporate an ecdysone-inducible construct, may be introduced into a suitable host cell by any appropriate transformation or transfection technique.

Preferably, the host cell will permit the expression of the NOS coding sequence. Thus, the cells may be chosen to be compatible with the said vector and may be for example bacterial, yeast, insect or mammalian cells. For NOS gene expression to be induced in the presence of ecdysone or an analog thereof, a cell harbouring an ecdysone inducible construct must preferably also be capable of expressing a functional ecdysone receptor.

As described above, the wild type *Drosophila* functional ecdysone receptor is

a heterodimer of the ecdysone receptor (EcR) and the product of the ultraspiracle gene (USP). Thus cells of the invention may be capable of expressing EcR and USP. However, replacement of EcR's natural heterodimeric partner USP with its mammlian homologue retinoid X receptor gives a heterodimer which can give more potent induction of an ecdysone responsive promoter. Thus cells of the invention may be capable of expressing EcR and RXR. It will be clear that cells of the invention may be capable of expressing functional variants of either subunit of the heterodimer. Functional variants of EcR and USP/RXR are polypeptides which can heterodimerise with their partner and can, when heterodimerised, allow ecdysone-responsive dimerisation to occur. In some cases functional variants may bind to non-wild type EcREs. Examples of functional variants and modified EcREs are described in No *et al. Proc. Natl. Acad. Sci. USA*, **93**: 3346-3351.

Preferred cells for use in the invention are human cells. Particularly preferred cells are EcR293 cells (Invitrogen, San Diego, CA; Catalogue No: R650-07; EcR293 is a derivative of the human fetal kidney cell line HEK293 (ECACC accession number 85/20602)). EcR293 cells are particularly suitable as they stably transformed with the vector pVgRXR. That vector is capable of expressing a functional variant of EcR, VgEcR, and RXR in mammalian cells and thus allows the expression of a functional ecdysone receptor. Other suitable cell lines include EcR-CHO and EcR-3T3 (Invitrogen, San Diego, CA; Cat. Nos: R660-07 and R680-07 respectively). Those two cell lines are stably transformed with the same vector, pVgRXR, as the EcR293 cell line.

Vectors of the invention, which incorporate an tetracycline-inducible construct, may be introduced into a suitable host cell by any appropriate transformation or transfection technique.

Preferably, the host cell will permit the expression of the NOS coding sequence in the presence of tetracycline or an analog thereof. Thus, the cells may be chosen to be compatible with the said vector and may be for example bacterial, yeast, insect or mammalian cells. For NOS gene expression to be regulated such that expression does not occur in the absence of tetracycline, a cell harbouring a tetracycline-inducible construct must preferably also be capable of expressing the

tetracycline repressor protein (tetR) or a functional variant thereof.

A functional variant of tetR is a polypeptide which is similar to tetR and which remains capable of binding, as a homodimer, the $TetO_2$ site or a functional variant thereof and tetracycline or an analog thereof. Typically, the binding affinity of a functional variant sequence of tetR for the $TetO_2$ site or a functional variant thereof or tetracycline or an analog thereof may be substantially the same as the binding affinity of the tetR polypeptide for the $TetO_2$ site or a functional variant thereof or tetracycline or an analog thereof. Alternatively, a functional variant sequence may have a binding affinity which may be greater or less than that of the tetR polypetide.

The *TetR* gene encodes a repressor protein of 207 amino acids with a calculated molecular weight of 23 kDa (Hillen and Berens, 1994, *supra*). A functional variant of tetR typically comprises an amino acid sequence substantially similar to that of the tetR sequence. Thus, a functional variant of a tetR will generally have at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98% or at least 99% sequence identity to tetR, calculated over the full length of those sequences. The calculation of sequence identities is described above.

A functional variant of the tetR sequence may be a modified version of that sequence obtained by, for example, amino acid substitution or deletion. Up to 1, up to 10, up to 20, up to 50, up to 75, up to 100 or more amino acid substitutions or deletions or combinations thereof may be made to the tetR sequence to produce a functional variant of that sequence. Substitutions are preferably made which result in a conservative amino acid substitution, for example as shown in the Table above.

Preferred cells for use in the invention are human cells. Particularly preferred cells are T-REx cells (Invitrogen, San Diego, CA; Catalogue Nos: R710-07, R712-07, R714-07 and R716-07). T-Rex cells are particularly suitable as they stably transformed with the plasmid pcDNA6/TR which generates high level expression of the tetR polypeptide. However, any cell line can be used which expresses tetR or a functional variant thereof.

Microcapsules

Cells of the invention may be encapsulated to give microcapsules. Preferably, microcapsules of the invention will be suitable for administration to a human or animal. Typically, microcapsules which are suitable for administration to a human or animal are biocompatible, immunoisolatory and mechanically stable.

Biocompatible microcapsules do not elicit a host response sufficient to result in rejection of the microcapsules or to render the microcapsules non-functioning. The term host responses includes all responses by a host to heterologous material, including for example immune responses such as cytotoxic and systemic responses, fibrotic responses, foreign body responses and other types of inflammatory responses induced by heterologous material. Biocompatibility may be determined by a combination of factors, including for example, the microcapsule material, surface texture and shape.

Materials used to form the microcapsules are thus typically selected for their ability to be accepted by a host harbouring the microcapsules. Suitable materials should generally be stable under *in vivo* physiological conditions and stresses. Typically, suitable materials will be stable *in vivo* over extended periods of time, for example up to 2 months, up to six months, up to 1 year or up to 2 years.

Suitable materials should also preferably be free from or have a content of leachable pyrogenic substances or other harmful, irritating or immunogenic substances. If the material to be used to form microcapsules is not free of those substances, it may be purified to remove the unwanted substances.

Preferred materials include reversibly and irreversibly gellable substances (for example, those that form hydrogels) and water-insoluble thermoplastic polymers.

Suitable hydrogel forming materials include alginates. Alginate is a linear copolymer composed of two monomeric units, D-mannuronic acid and L-guluronic acid. Preferred alginates are those low in or free from toxic substances, pyrogens and immunogenic materials such as proteins and complex carbohydrates, for example highly purified high-G alginates.

Alginates may be induced to form gels in the presence of certain divalent cations, for example Ca^{2+}. The major limitation to the use of Ca^{2+} alginate as a

microcapsule material is its sensitivity to chelating compounds such as phosphate, citrate and lactate or anti-gelling cations such as Mg^{2+} or Na^{2+}. To overcome this limitation alginate gels can be stabilized by replacing Ca^{2+} with other divalent cations having a higher affinity for alginate. The affinity series for various divalent caitons is, in order of decreasing affinity for alginate: $Pb^{2+} > Cu^{2+} > Cd^{2} > > Ba^{2+} > Sr^{2+} > Ca^{2+} > Co^{2+} = Ni^{2+} = Zn^{2+} > Mn^{2+}$. It has been shown that the rigidity of alginate gels in general increases with the affinity (though Cd^{2+} and Ni^{2+} do not fit this rule): $Pb^{2+} > Cu^{2+} = Ba^{2+} > Sr^{2+} > Cd^{2+} > Ca^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+} > Ni^{2+}$. However, the high toxicity of most of these ions, in particular Pb^{2+}, Cu^{2+} and Cd^{2+}, means that use for microcapsules suitable for administration to a human or animal is strictly limited. In pratise only Sr^{2+} and Ba^{2+} are likely to be suitable for encapsulation of living cells. Alginate gels may also be stabilized by adding other multivalent ions such as Ti^{3+} and Al^{3+}.

Alginate forms strong complexes with polycations such as chitosan or polypeptides, or synthetic polymers such as polyethylenimine. These complexes do not dissolve in the presence of Ca^{2+} chelators or anti-gelling cations and may therefore optionally be used both to stabilize the gel and to reduce the porosity. Additionally, covalent crosslinking may be used to stabilize alginate gels. Covalent crosslinking may be carried out by techniques such as direct crosslinking of the carboxyl groups and covalent grafting of alginate with synthetic polymers. This gives gels with improved stability and mechanical strength. However, these techniques involve reactive chemicals and thus may be difficult to apply to the encapsulation of living cells.

Suitable thermoplastics are typically those which are mildly hydrophobic, for example those having a solubility parameter of from 8 to 15 $(joules/m^3)^{½}$ or more preferably from 6 to 14 $(joules/m^3)^{½}$ [Solubility parameter as defined in Polymer Handbook 3rd Ed., John Wiley and Sons, NY] . Suitable polymers are selected to have a solubility parameter low enough so that they are soluble in organic solvents, but high enough so that they may partition to from a membrane. Such polymers should be free from labile nucleophilic moieties and be resistant to oxidants and enzymes even, for example, in the absence of stabilizing agents.

Examples of suitable thermoplastic polymers are polyacylonitrile vinyl chloride (PAN/PVC), polyacrylonitrile (PAN), polymethylmethacrylate (PMMA), polyvinyldifuoride (PVDF), polyethylene oxide, polyolefins (eg. polyisobutylene or polypropylene), polysulfones or cellulose derivatives (eg. cellulose acetate or cellulose butyrate).

The microcapsule forming material may also comprise one or more substance which reduces or eliminates local host inflammatory responses to the microcapsules and/or stimulates the formation of a suitable environment for the microcapsules. For example, an antibody to a mediator of the immune response could be included. Suitable antibodies include antibodies to the lymphokines, tumour necrosis factor (TNF) and interferon (IFN). An anti-inflammatory steroid may be included in the microcapsule forming material. Also, a substance which stimulates angiogenesis (ingrowth of capillary beds) can be included. This may be desirable where the isolated cells or tissues are required to be in close contact with the host bloodstream for proper functioning. Cells which are encapsulated may themselves be capable of generating any of the substances mentioned in this paragraph.

The texture of the exterior of the microcapsules is typically formed so after implantation it provides an optimal interface with the tissues of the host. The appropriate texture will in part be determined by the site of implantation. For example, if the microcapsules are to be introduced into the intraperitoneal cavity of the host, its surface should be smooth. A smooth surface may discourage excessive overgrowth by fibroblasts. The overgrowth of fibroblasts may be disadvantageous as it may result in the deposition of a poorly permeable basement membrane around microcapsules and consequent isolation of microcapsules from the host. However, if the microcapsules are to be embedded in the soft tissues of the host the surface may be moderately rough or stippled. A factor to be taken into account is whether it is desired for host cells to attach to the surface of the microcapsules or alternatively if such contact is to be avoided.

Microcapsules of any shape may be formed. However, certain microcapsule shapes may elicit fibrotic responses and therefore those shapes are typically avoided. Thus microcapsules preferably should not comprises structures having interlayers

such as brush surfaces or folds. In general, opposing microcapsule surfaces or edges either from the same or adjacent microcapsules should be at least 2mm apart, more preferably at least 5mm apart. Preferred shapes for microcapsules are thus, substantially spherical, substantially cylindrical, U-shaped cylinders and flat sheets or sandwiches. Substantially spherical microcapsules may be formed and offer the advantage that delivery of cells so encapsulated may be carried out by intraperitoneal injection. This may be advantageous if systemic delivery is required. Cylinders, by contrast, generally require surgical implantation, but as described below, may have advantages in terms of surface area to volume ratios.

Microcapsules of the invention are typically immunoisolatory. Immunoisolatory microcapsules protect encapsulated cells from the host immune system. The encapsulating material may protect encapsulated cells by: (1) substantially preventing or minimising components of the host foreign body response mechanisms from entering a microcapsule; (2) providing a barrier which substantially prevents or minimises contact between the encapsulated cells and components of the host immune system; and (3) providing a barrier which substantially prevents or minimises contact between inflammatory. antigenic or other harmful substances that may be present in a microcapsule and components of the host foreign body response mechanisms.

Thus the microcapsule material will generally allow passage of substances up to a predetermined size, but prevent the passage of larger substances and therefore microcapsules are differentially permeable. The molecular weight cutoff (MWCO) selected for particular microcapsules may be determined in part by (1) the type and extent of immunological rejection anticipated after introduction of microcapsules to the host; and (2) the molecular size of the largest substance whose passage into and out of the microcapsules is required.

The type and extent of immunological rejection encountered by microcapsules may depend in part on the type of genotype of the encapsulated cells and the genotype of the host. When the encapsulated cells are allogenic to the host rejection may proceed largely through cell-mediated attack by the host's immune cells against the implanted cells. When the encapsulated cells are xenogenic to the

recipient, rejection through assembly of the host's cytolytic complement attack complex may predominate, as well as antibody interaction with complement.

The MWCO of the encapsulation material must therefore be sufficiently low to prevent access of the molecules required to carry out the above mentioned rejection mechanisms to the encapsulated cells, yet sufficiently high to allow passage of any therapeutic substance produced by the encapsulated cells. For example, materials can be used which allow passage of molecules up to about the size of C1q (~400kd), the largest polypeptide required for the assembly of the cytolytic complement attack complex. Alternatively, it is possible to use microcapsule materials which allow passage of molecules up to the size of immunoglobulin G (~150kd) and exclude larger molecules. Preferably, microcapsules of the invention may have a low MWCO. NO is a small molecule and thus microcapsules of the invention can have a low MWCO, which will in turn increase immunoisolation of the encapsulated cells.

Diffusion of small molecules appears unaffected by alginate gels, whereas passage of larger molecules may be restricted. The highest diffusion rates of proteins, indicating the most open pore structure, are found in beads made from high-G alginates. Several procedures for stabilizing alginate gels also influence porosity. For example, the formation of poly-anion-polycation membranes with polypeptides or chitosan can be used to prevent diffusion of antibodies from microcapsules. By controlling the molecular weight of the polycations, pore sizes below selected cut-off values can be obtained. An example of a suitable polycation is poly-L-lysine; the lower the molecular weight M_r of the poly-L-lysine, the less permeable the capsules.

Contact between components of the host response mechanisms and encapsulated cells and other inflammatory, antigenic or other harmful substances may be substantially prevented or minimised of the microcapsule material forms a physical barrier between those substances. The thickness of the barrier may vary, but typically it should be sufficiently thick to prevent direct contact between substances on either side of the barrier. Thus the thickness of the barrier generally ranges from 5 to 200μm, preferably from 10 to 100μm and most preferably from 20 to 50μm.

Microcapsules of the invention may harbour from 1 to at least 100 cells, at

least 200 cells, at least 500 cells, at least 1000 cells or at least 1 x 10^4 cells, up to 1 x 10^5 cells or up to 1 x 10^7 cells. Factors which influence the number of cells that a microcapsule may harbour include: (1) microcapsule size and shape; (2) mitotic activity within the microcapsule; and (3) viscocity of the interior of the microcapsule.

In relation to the first of these factors, microcapsule size and shape, the diffusion of nutrients into cells and of waste products out of cells is critical to the continued viability of the encapsulated cells. Since diffusion in and out of the microcapsule may be limited by the microcapsule surface area, the surface to volume ratio of microcapsules will be critical in determining how many cells may be maintained within the microcapsules.

Among the nutrient requirements of encapsulated cells is that of oxygen. The oxygen requirement of cells must be determined for any cell type of choice. However, a typical example may be that for cells encapsulated in a cylindrical microcapsule of 900μm outer diameter implanted in the intraperitoneal space ($pO_2 \approx$ 45-50mmHg) the optimal total cell volume is in the range of up to 20%, preferably 1 to 15% and most preferably about 5% of the microcapsule volume. For a cylindrical microcapsule of 400μm outer diameter the optimal cell volume may be from 30 to 65% total microcapsule volume and preferably may be 35%.

At implantation sites where the partial oxygen pressure is less than the peritoneum, lower loading densities may be required. Implantation into an artery ($pO_2 \approx$ 95mmHg), by contrast, will allow support of a greater cell volume per microcapsule.

If the cells used actively divide in the microcapsule, then they will continue to divide until they fill the available space, or until phenomena such as contact inhibition limit further division. Thus, when dividing cells are used the size and shape of the microcapsules may be chosen so that complete filling of the microcapsule will not lead to a loss of nutrient passage to cells because of diffusion limitations. Typically, microcapsules that will be filled to confluency with cells will be no more than 250μm in cross-section, such that cells in the interior will have less than 15 between them and an external surface, preferably less than 120 cells and more preferably less than 5 cells.

Cells present in densities of up to 70% of the microcapsule volume can be viable, but cell solutions in this concentration range would have considerable viscosity. Thus formation of microcapsules with such high cell densities may be difficult. Generally, cell densities of 30% or less, or 20% or less will be appropriate for microcapsule formation.

Microcapsules are preferably substantially spherical or substantially cylindrical. They may be of any appropriate size, but the size will be influenced by a number of factors including some of those mentioned above. For example, nutrient diffusion may limit microcapsule size. Substantially spherical microcapsules may be for example from 0.01 to 4.0 mm, preferably from 0.1 to 3.0 mm and more preferably from 0.2 to 2.0 mm in diameter. Substantially cylindrical microcapsules may be from 0.01 to 4.0 mm, preferably from 0.1 to 3.0 mm and more preferably from 0.2 to 2.0 mm in outer diameter. Such cylindrical microcapsules may be for example from 0.1 mm, 0.2 or 0.5 mm in length to for example 5 mm, 10 mm or 20 mm in length. For a population of microcapsules, the above dimensions may be average dimensions for the population.

Microcapsule formation

Microcapsules may be produced according to methods well known to those skilled in the art.

Alginate microcapsules may be formed, for example, by mixing an alginate solution with a cell suspension and then dripping the alginate-cell suspension into a solution containing Ca^{2+} ions. A poly-L-lysine layer may be added by to the microcapsules thus formed by incubation in a solution of poly-L-lysine. The poly-L-lysine layer may increase mechanical stability and decrease permeability of the resulting microcapsules. A final alginate layer may be added by a further incubation in an alginate solution.

Typically an initial sodium alginate solution at a concentration of from 1.5 to 12%, more preferably from 2 to 4% is prepared by dissolving an appropriate amount of Na^{+}-alginate in distilled water or buffer. The suspension may be stirred for 6 hours by a magnetic stirrer or left overnight on a rotary shaker at room temperature.

The resulting suspension may be filter sterilized by use of a 0.22μm membrane filter.

Cells may then be mixed with the Na^+-alginate solution. If the cells are added as a suspension in a buffer, the presence of phosphate, citrate, EDTA and divalent cations should be avoided. Microcapsules may formed by dripping the alginate-cell suspension (from a syringe with an inner diameter of for example from 0.22 to 1.0mm, at a distance of for example about 20cm away from the $CaCl_2$ surface) into a solution containing for example from 20 to 100mM Ca^{2+} ions. The size of the microcapsules may be controlled by the application of a coaxial airstream. For a given Na^+-alginate solution (concentration, type and intrinsic viscosity) the size of the microcapsules depends only on the airflow for a given syringe diameter and microcapsules of diameters in the range of, for example, from 0.2mm to 3mm may be produced. The microcapsules may be left to harden in the $CaCl_2$ for from 5 to 30 minutes, depending on their diameter.

The resulting microcapsules may be washed in saline or a suitable buffer and then suspended in a solution of poly-L-lysine. The poly-L-lysine used may be a molecular weight of for example from 24, 000 to 65, 000, preferably 27, 000 and the microcapsules may typically be suspended in a 0.05% (w/v) solution of poly-L-lysine. After another washing step with saline or a suitable buffer, the microcapsules may be suspended in a Na^--alginate solution (at a concentration of for example, 0.15%). The microcapsules may further be suspended in a solution of sodium citrate (at a concentration of for example, 55mM). The microcapsules are generally given a further saline wash before use.

Cylindrical microcapsules can be formed by loading thermoplastic polymer hollow fibres, such as polyethersulfone hollow fibers, with a cell suspension and sealing the ends of the hollow fibre.

Newly-formed microcapsules may be maintained until use under sterile conditions in a non-pyrogenic, serum-free defined nutrient medium or balanced salt solution at about 37°C until implantation. Alternatively the microcapsules may be cryopreserved.

Therapeutic Use

Microcapsules of the invention may be used to treat conditions which are associated with deficient NO production. Microcapsules may also be used for the manufacture of a medicament for use in the treatment of a condition associated with deficient NO production. Conditions associated with deficient NO production include those that may be a result of abnormal deficient NO production and also those that although not a result of abnormal deficient NO production as such, may be treated by raising NO levels. The condition of a patient suffering from such an infection can be improved by administration of an inhibitor. A therapeutically effective amount of an inhibitor may be given to a human patient in need thereof.

Microcapsules of the invention may therefore be used to treat, for example, hyperlipidaemia, renal failure, hypertension, restenosis after angioplasty, atherscloerosis and its complications, complications of heart failure or schizophrenia.

NO is known to play an important role in the host defence against tumors. Rodent macrophages activated by IFN-γ and IFN-α produce large quantities of NO, which has been shown to kill certain tumor cells, including human ovarian cancer, leukaemic, colon cancer and prostate cancer cells. By contrast, human macrophages produce a much lower level of NO and have probably have a limited tumor killing ability. Therefore, microcapsules of the invention may be used as artificial macrophages in the treatment of cancers.

Microcapsules may be administered to a human or animal in need thereof and ecdysone or an analog thereof administered subsequently. Alternatively, ecdysone or an analog thereof may be administered at the same time as the microcapsules. Thus the invention provides a product containing microcapsules of the invention and ecdysone or an analog thereof as a combined preparation for simultaneous, separate or sequential use in the treatment of a condition associated with deficient NO production.

Implantation of microcapsules is typically performed under sterile conditions. Generally, the microcapsules are implanted at a site in the host which (1) allows for appropriate delivery of NO produced by the microcapsules to the host; and (2) allows for passage of nutrients to the encapsulated cells. Also the site may be chosen to allow for the recapture of the microcapsules.

Microcapsules may be administered in a variety of dosage forms. Thus, they can be administered orally, for example as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules. The inhibitors may also be administered parenterally, either subcutaneously, intravenously, intramuscularly, intrasternally, transdermally or by infusion techniques. The microcapsules may also be administered as suppositories. A physician will be able to determine the required route of administration for each particular patient.

The formulation of microcapsules for use in preventing or treating a condition associated with deficient NO production will depend upon factors such as the nature of the microcapsules, whether a pharmaceutical or veterinary use is intended, etc.

Microcapsules are typically formulated for administration in the present invention with a pharmaceutically acceptable carrier or diluent. The pharmaceutical carrier or diluent may be, for example, an isotonic solution. For example, solid oral forms may contain, together with the microcapsules, diluents, e.g. lactose, dextrose, saccharose, cellulose, corn starch or potato starch; lubricants, e.g. silica, talc, stearic acid, magnesium or calcium stearate, and/or polyethylene glycols; binding agents; e.g. starches, arabic gums, gelatin, methylcellulose, carboxymethylcellulose or polyvinyl pyrrolidone; disaggregating agents, e.g. starch, alginic acid, alginates or sodium starch glycolate; effervescing mixtures; dyestuffs; sweeteners; wetting agents, such as lecithin, polysorbates, laurylsulphates; and, in general, non-toxic and pharmacologically inactive substances used in pharmaceutical formulations. Such pharmaceutical preparations may be manufactured in known manner, for example, by means of mixing, granulating, tabletting, sugar-coating, or film coating processes.

Liquid dispersions for oral administration may be syrups, emulsions and suspensions. The syrups may contain as carriers, for example, saccharose or saccharose with glycerine and/or mannitol and/or sorbitol.

Suspensions may contain as a carrier, for example a natural gum, agar, sodium alginte, pectin, methylcellulose, carboxymethylcellulose, or polyvinyl alcohol. The suspensions or solutions for intramuscular or intratumor injections may contain, together with the microcapsules, a pharmaceutically acceptable carrier, e.g. sterile water, olive oil, sesame oil, ethyl oleate, glycols, e.g. propylene glycol, and if

desired, a suitable amount of lidocaine hydrochloride.

Solutions for intravenous administration or infusions may contain as carrier, for example, sterile water or preferably they may be in the form of sterile, aqueous, isotonic saline solutions.

A therapeutically effective amount of microcapsules is administered to a patient. The dose of microcapsules may be determined according to various parameters, especially according to the microcapsules used; the age, weight and condition of the patient to be treated; the route of administration; the required regimen; and the condition to be treated. Again, a physician will be able to determine the required route of administration and dosage for any particular patient. A typical dose is from about 1mg/kg to 10mg/kg of body weight, according to the activity of the microcapsules, the age, weight and conditions of the subject to be treated, the type and severity of the degeneration of the microcapsules and the frequency and route of administration.

Ecdysone or an analog thereof or tetracycline or an analog thereof may be administered simultaneously, separately or sequentially with microcapsules of the invention. Ecdysone or an analog thereof or tetracycline may be administered and formulated for administration, for example as described above for microcapsules of the invention. The amount of ecdysone or tetracycline required and the frequency of admininstration will depend on similar considerations as apply to microcapsule dosages. It may be necessary to give several doses of ecdysone or tetracycline over a period of time or alternatively, it may only be necessary to administer a single dose at the beginning of the treatment.

Ecdysone or an analog thereof or tetracycline or an analog thereof can itself be microencapsulated, for example by a method for microencapsulation as described above. Such microcapsules could be administered along with microcapsules of the invention. Thus, the invention provides a product containing microcapsules of the invention and microcapsules harbouring ecdysone or an analog thereof or tetracycline or an analog thereof as a combined preparation for simultaneous, separate or sequential use in the treatment of a condition associated with deficient NO production. Ecdysone or tetracycline may also be incorporated into the material used

to encapsulate cells of the invention. Encapsulation of ecdysone or an analog thereof or tetracycline or an analog thereof or incorporation of ecdysone or tetracycline into the encapsulation material may abrogate the need for more than one administration of ecdysone or an analog thereof or tetracycline or an analog thereof.

The following Examples illustrates the invention:

Examples

Materials and methods

Unless indicated otherwise, the methods used are standard biochemical techniques. Examples of suitable general methodology textbooks include Sambrook *et al.*, Molecular Cloning, a Laboratory Manual (1989) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.

Subculture Procedure

All of the operations were carried out under strict aseptic conditions in a laminar flow hood. The medium was removed from near-confluent flasks and gently washed twice with serum-free DMEM (Dulbecco's Modified Eagle's Medium). A trypsin/versene mix was added to the cells and they were incubated for 5-10 min. until the cells had detached. Once the cells had detached they were resuspended in pre-warmed (37°C) serum-free DMEM and then pelleted by centrifugation at 1000rpm for 5min. It was essential to wash the cells to remove the residue of the tyrpsin/versene (serum-free DMEM was used for washing). The supernatant was removed and the cells gently resuspended in 15ml of 90% DMEM with glutamine, 10% fetal bovine serum (FBS, cell culture grade) that had been pre-warmed to 37°C. The cells were then transferred to three or four T-25 flasks (or equivalent) and placed in a humidified, 37°C, 5% CO_2 incubator. It typically took 3-4 days for cell cultures to reach 80-90% confluency. Media were changed 2 times a week and cells subcultured at a ratio of 1:3 to 1:4 when they reached 80-90% confluency. Cells were subcultured when approaching confluency to avoid the accumulation of floating and dead cells. Cells were frozen for storage in 95% FBS and 5% DMSO.

Induction using Muristerone A

Cells could be maintained in non selection medium for 2-3 weeks without losing inducibility following muristerone A treatment. Non-selection medium was used by choice, although selection medium worked equally well. Cells were seeded at 1-2 x 10^5/ml concentration for 12 well or 96 well plates. Semi-confluent or confluent plates or flasks were used for induction. Figure 1(a) shows overnight induction in 12 well plates (3-30 hours) following the addition of different doses of muristerone A. Nitrate concentrations are measured using the Griess Reaction (see below).

Western blot analysis

Cells were pelleted at 200g, followed by two washes in ice-cold PBS, pH 7.2, then resuspended in the extraction buffer (50mM NaF, 20mM Hepes (pH 7.8), 450mM NaCl, 25% (vol/vol) glycerol, 0.2mM EDTA, 0.5mM dithiothreitol, 0.5mM phenylmethylsulfonyl fluoride, leupeptin (0.5µg/ml), protease inhibitor (0.5µg/ml), trypsin inhibitor (1.0µg/ml), aprotinin (0.5µg/ml), bestatin (40µg/ml)) and left on ice for 10 min. Following centrifugation at 10,000g for 10 min at 4°C, the supernatant was collected and the cell extract assayed for protein using the BCA kit (Pierce). One fifth of a volume of five times sample buffer (0.25M Tris-HCL (pH6.8), 0.4M DTT, 5% SDS, 0.5% bromophenol blue, 50% glycerol) was added to each sample and boiled for 5 min prior to storage at -70°C. Electrophoresis was carried out on 6% SDS polyacrylamide gels with 25µg samples. Proteins were transferred to polyvinylidine difluride (PVDF) membranes (Amersham) and immunoblotting carried out with the appropriate antibody using ECL (Amersham). Where necessary, blots were stripped in 62.5mM Tris-HCL 100mM β-mercaptoethanol/ 2% SDS, (pH 6.7) and reprobed with different antibodies.

Northern Blotting

Poly(A) + mRNA was isolated using a micro-fastrack mRNA purification kit (Invitrogen), separated by electrophoresis and transferred onto Hybond N membrane (Amersham). Phosphoimaging (BAS1000, Fujix) was used to quantify the signals

using the MacBas image analysis software.

NOS activity assay

Griess Reaction (Green *et al.*, 1982, Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids, *Anal. Chem.* **126**, 131-138): NOS activity was determined for both intact cells and their lysates. For intact cells, 100µl of the culture medium was mixed with 100µl of Griess reagent (1:1 mixture of 1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethyethylenediamine dihydrochloride in water) for 10 min at room temperature and the absorbance at 543 nm was recorded. A serial dilution of sodium nitrite was used as a standard.

For enzyme assays on cell lysates, 100µg of lysate was mixed with 100µl reaction reagent from the NOS detect system (Stratagene NOS detect kit, Cat. No. 204500). The kit measures the conversion of [^{14}C]arginine to [^{14}C] citrulline, and is specific for the NOS pathway.

Example 1 - Generation of NOS transfected cell lines under the control of an ecdysone-responsive or a tetracycline-responsive promoter

Three plasmids were generated, each of which expressed one of the human NOS isoforms under the control of an ecdysone-responsive promoter. An additional plasmid was constructed placing the iNOS cDNA under the control of a tetracycline-regulated promoter.

(I) pIND-hiNOS-f (Figure 1a)

4164bp of the human iNOS cDNA (GenBank accession number: X73029, Coding sequence 226-3687) was cut from its original vector (Bluescript KS) using the restriction endonucleases, *Kpn*I and *Spe*I and cloned into pIND (Invitrogen, San Diego, CA; Catalogue No: V705-20) which had been cut with *Kpn*I and *Xba*I. The resulting plasmid was sequenced to confirm that the cDNA had inserted in the correct orientation.

(II) pIND-hnNOS-f (Figure 1b)

5kb of the human nNOS cDNA (GenBank accession number: U17327, Coding sequence 686-4990) was cut from its original vector (Bluescript KS) using the restriction endonucleases, *Xba*I and *Kpn*I and cloned into pIND (Invitrogen, San Diego, CA; Catalogue No: V705-20) which had been cut with *Nhe*I and *Kpn*I. The resulting plasmid was sequenced to confirm that the cDNA had inserted in the correct orientation.

(III) pIND-heNOS-f (Figure 1c)

The wild type human eNOS (GenBank accession number: M95296, Coding sequence 21-3632) was cut from its original vector (Bluescript KS) using the restriction endonucleases, *Hind*III and *Not*I and cloned into pIND (Invitrogen, San Diego, CA; Catalogue No: V705-20) which had been cut with *Hind*III and *Not*I. The resulting plasmid was sequenced to confirm that the cDNA had inserted in the correct orientation.

(IV) pTet-hiNOS-f (Figure 1d)

The human iNOS cDNA (GenBank accession number: X73029, Coding sequence 226-3687) was cut from its original vector (Bluescript KS) using the restriction endonucleases, *Kpn*I and *Spe*I and cloned into pcDNA4/TO (Invitrogen, San Diego, CA) which had been cut with *Kpn*I and *Xba*I. The resulting plasmid was sequenced to confirm that the cDNA had inserted in the correct orientation.

The pIND plasmid contains 5 modified EcREs called E/GREs which bind a modified functional ecdysone receptor. That modified functional ecdysone receptor can be expressed by another plasmid, pVgRXR (Invitrogen, San Diego, CA; Catalogue Number: V730-20). pVgRXR constitutively expresses a heterodimeric receptor comprising a modified ecdysone receptor (VgEcR) and RXR. Thus, a cell transformed with pVgRXR and one of the three plasmids described above (I, II or III) will express NOS in the presence of ecdysone or an analog thereof. In the presence of ecdysone the functional ecdysone receptor binds to the E/GREs and transcription of the NOS cDNA is initiated.

The plasmid pIND-hiNOS-f was used to transfect a human fetal kidney cell line, EcR293 (Invitrogen, San Diego, CA; Catalogue No: R650-07), which is stably transformed with pVgRXR. Transfections were carried out using Superfect reagent (Qiagen) and transfectants were isolated following double selection on G418 (400μg/ml) and zeocin (250μg/ml) for 14 days.

Thus, cells were isolated which constitutively expressed the subunits of a functional ecdysone receptor, RXR/VgEcR and the human iNOS cDNA under the control of an ecdysone-inducible promoter.

The T-Rex system (Invitrogen, San Diego, CA; Catalogue No: K1020-01) is a tetracycline-regulated mammalian expression system that uses regulatory elements from the *E. coli* Tn*10*-encoded tetracycline resistance operon. The pcDNA4/TO plasmid allows expression of a gene of interest under te control of the strong human cytomegalovirus immediate-early (CMV) promoter and two tetracycline operator 2 ($TetO_2$) sites. The pcDNA6/TR plasmid expresses high levels of the *Tet*R gene under the control of the human CMV promoter. Thus, a cell transformed with pcDNA6/TR and the plasmid described above (IV) will express NOS in the presence of tetracycline or an analog thereof. When present, tetracycline binds to tetR which undergoes a conformational change such that it dissociates from the $TetO_2$ sites. Expression of the iNOS gene is then induced, driven by the CMV promoter.

The plasmid pTet-hiNOS-f was used to transfect the cell line T-REx-293 ((Invitrogen, San Diego, CA; Catalogue No: R710-07). The T-REx-293 cell line is a human embryonic kidney 293 cell line which has been transfected with the pcDNA6/TR plasmid and thus generates high level expression of the tetracycline repressor protein (TetR). Transfections were carried out using conditions as described above for the muristerone A inducible constructs. Transfectants were isolated following selection on zeocin (200μg/ml) for the human iNOS cDNA expressing plasmid and blasticidin (5μg/ml) for the tetR expressing plasmid.

Thus, cells were isolated which constitutively expressed the tetracycline repressor protein (tetR) and the human iNOS cDNA in the presence of tetracycline.

Example 2 - Isolation and characterization of ecdysone-responsive and tetracycline-responsive human cell lines

(A) Isolation of a panel of ecdysone-responsive human cell lines and determination of NOS activity

A panel of 20 G418/zeocin-resistant clones were examined for their ability to generate NO. A total of 5 were identified that could be induced to produce NO at varing levels after treatment with 100μM muristerone A for 24 hours. NOS activity was determined in both intact cells and in cell lysates. For intact cells, the Griess reaction was used to determine the concentration of NO in 100μl of culture medium. For enzyme assays, 100μg of cell lysate was mixed with 100μl reaction reagent from NOS detect system (Stratagene NOS detect kit, Cat. No.204500). The kit measures the conversion of [^{14}C] arginine to [^{14}C] citrulline, and is specific for NOS. For a typical experiment, transfectants were plated out on 12 well Falcon tissue culture plates at a cell density of 1 x 10^5/ml and nitrite was measured by the Griess reaction. Muristerone A (Invitrogen) was added at a final concentration of 100μM to specific wells, and after induction for 24hr, 100μl of culture supernatant was used to measure nitrite concentration using the Griess reagent. The results are reported as the average of assays run on triplicate wells. Well-to-well variation was less than 10%.

(B) Time and dose response of EcR293 clone 11 cells generating NO

One of the transfectants, clone 11, was selected for further study. Cells were grown with varying concentrations of muristerone A, and at different time intervals, supernatants were taken and the Griess reaction was used to measure the nitrite concentration. The results are reported as the average of assays run in triplicate. Well-to-well variation was less than 10%. See Figure 2a.

(i) Muristerone A-dependent expression of the human iNOS gene. Northern blot analysis was carried out with 2μg of polyA+ RNA isolated from cells which had been treated with muristerone A for 24 hr. A human iNOS cDNA probe was used to detect the presence of a 4kb band in mRNA extracted from cells treated with either 1μM or 10μM muristerone A. Human β-actin mRNA was used as a loading control. See Figure 2b.

(ii) Western blot of iNOS protein expression was carried out on untreated control cells or cells treated with 10μM muristerone A. The cells were harvested and 20μg of whole cell extracts loaded on to a 6% polyacrylamide gel. Following electrophoresis, the proteins were transferred to a filter and probed with a polyclonal antibody raised against the 7 C-terminal residues of human iNOS: Cyc-Arg-Nle-Orn-(Ser-Leu-Glu-Met-Ser-Ala-Leu). Filters were stripped and an antibody against human alpha-tubulin. (Insight Biotchnology) was used as a control. See Figure 2b.

(C) Isolation of a panel of tetracycline-responsive human cell lines and determination of NOS activity

A panel of 4 tetracycline-regulated iNOS expressing cell lines were isolated, designated clone 1, clone 2. clone 5 and clone 22.

In the absence of the inducer tetracycline the iNOS transfectants were unable to express mRNA as the two Tet operator sites ($Tet0_2$) are occupied by the repressor protein effectively blocking transcription. When tetracycline is added to the culture medium, it binds to the TetR protein and changes its conformation. The altered conformation of the repressor is unable to bind the operator sites, and consequently iNOS can be expressed. Nitrite concentrations were determined using the Griess reaction (see figure 3).

Example 3 - Encapsulation of recombinant EcR293 clone 11 cells

EcR293 clone 11 cells were used for encapsulation studies. Prior to encapsulation, EcR293 clone 11 cells were maintained in Dulbecco's media supplemented with 10% fetal bovine serum (FBS) and penicillin (100 units/ml) and streptomycin (100μg/ml) in the absence of both G418 and zeocin. The cells were encapsulated in alginate-poly-L-lysine-alginate microcapsules as follows:

Cells were harvested with trypsin then resuspended in a 1.8% sodium alginate solution (Sigma, catalogue no. A2033, medium viscosity, filter sterilised through a 0.25μm membrane) in PBS at between 0.5-5 x 10^7 cells/ml. The suspension was placed in a syringe and held 20 to 40cm above a beaker containing an excess (100ml) of stirred ice cold calcium chloride solution (0.2M, pH7, filter sterilized). The cell

suspension was extruded through a 21G needle and droplets made to fall into the solution of sterile ice cold calcium chloride. Upon contact with the calcium chloride buffer, alginate gelation is immediate and the cells are encapsulated in an alginate complex. The microcapsule size was controlled by the extrusion pressure and the needle gauge. A typical hypodermic needle produced beads of from 0.5 to 2mm in diameter. Other shapes could be obtained by using a mould the wall of which was permeable to calcium ions.

Droplets were allowed to gel for between 1 to 30 minutes and were then suspended for 1 to 10 minutes in a 1:1 mixture of poly-L-lysine and 1.8% sodium alginate solution. The positively charged polylysine forms a complex with the alginate to form a semipermeable membrane. Immediately following encapsulation and washing, the cells were maintained in Dulbecco's media (supplemented with 10% fetal bovine serum (FBS) and penicillin and streptomycin) or PBS prior to further experimentation.

The microencapsulation procedure is illustrated schematically in Figure 4.

Example 4 - *In vitro* culture of the encapsulated cells

When EcR293 clone 11 cells were encapsulated with immunoisolating microcapsules fabricated from alginate-poly-L-lysine-alginate, the cells survive for at least 3 days. Moreover, the encapsulated cells could be induced to produce nitric oxide at a controlled level upon stimulation with muristone A at a concentration of 10μM..

When muristerone A was removed, the NO level dropped. The cells could be "switched on" again when muristerone A was reapplied at a concentration of 10μM (see Figure 5).

Example 5 - *In vivo* injection and retrieval of the encapsulated cells

Male CT-1 mice (3-4 weeks old, about 15 grams body weight), were intraperitoneally injected with 1ml of microcapsules from Example 3 with a 16G catheter. The inducer, 1mg per mouse of muristerone A, was added at the same time. Two days later, a full midline incision of the abdominal skin and a 0.75cm incision

through the abdominal wall were made and the microcapsules were retrieved from the peritoneal cavity.

The cells were washed three times with ice-cold phosphate-buffered saline (PBS) and then supplemented with Dulbecco's media. The capsules were then tested for survival and inducibility by muristerone A. The test showed that the microencapsulated cells were still capable of induction by 10μM muristerone A and could produce nitric oxide accordingly.

It is thus feasible that this type of microencapsulated cell line could be used as an artificial endothelial cell or macrophage for human disease treatment.

Example 6 - Microencapsulated cells as an artificial macrophage for treating human ovarian and colon cancer

A nude mouse/xenograf model was used to assess the ability of microencapsulated cells to kill tumor cells *in vivo*. The present studies utilized two human carcinoma cell lines that produce rapidly growing subcutaneous tumors in nude mice were used. DLD-1 is a human colon adenocarcinoma cell line, while SK-OV-3 is an ovarian carcinoma cell line. Both were obtained from ATCC. The cell lines were maintained as adherent monolayers in DMEM supplemented with 10% FBS. Cultures were maintained on plastic and incubated in 5% CO_2-95% air at 37°C. All cultures were free of *Mycoplasma*.

For all *in vivo* experiments, tumor cells in the exponential phase were harvested by a 1 to 2 min treatment with 0.25% trypsin, 0.02% EDTA solution (w/v). The flasks were tapped sharply to dislodge the cells, DMEM added, and the cell suspension pipetted to produce a single-cell suspension. The cells were washed, resuspended in $Ca2^+/Mg2^+$-free PBS and diluted to the desired concentration. Cell viability was determined by trypan blue exclusion, and only single-cell suspensions of greater than 95% viability were used for *in vivo* experiments.

To produce subcutaneous tumors in female CD1 nude mice, suspensions of tumor cells in 1ml of PBS were mixed with 1ml of alginate encapsulated EcR293 clone 11 cells. To induce NO production, 10mg of ponasterone A was dissolved into 1ml of sesame oil. The ponasterone A solution was mixed with the tumor

cell/encapsulated cell mixture. In control experiments 1ml of sesame oil was used in the absence of ponasterone A.

A total volume of 300µl of the cell suspension/mixture was injected (with a 21G needle) subcutaneously into the lateral flank proximal to the midline of the unanaesthetized animal. Each experiment used between 5 and 10 animals. The mean diameter of the tumors was determined by measuring tumor length (l) and width (w) by calipers. Tumor volume was estimated by using the formula-tumor volume = length x width^2 x 2.54. Tumors obtained from each of the experiments were either frozen in liquid nitrogen or subsequent analysis by northern or western blotting or for histological evaluation. The above procedure was carried out with both of the human carcinoma lines.

Colon cancer DLD-1 cells ($1x10^6$ per mouse) were injected subcutaneously along with microencapsulated EcR293 clone 11 cells; $5x10^6$ cells microencapsulated as described in Example 3 above. The microencapsulated cells were injected in the presence of either sesame oil alone or sesame oil and ponasterone A (1mg per mouse). The results are shown in Figure 6a and demonstrate that the microencapsulated cells plus ponasterone A treatment slowed the growth of DLD-1 cells considerably compared to the treatment of microencapsulated cells alone.

The experiment was repeated with SK-OV-3 ovarian cancer cells. Encapsulated EcR293 clone 11 cells, $3x10^6$ cells per mouse microencapsulated as described in Example 3 above, were introduced into mice with 5×10^5 SK-OV-3 cells. The encapsulated cells were introduced in either in the presence of sesame oil alone or sesame oil containing ponasterone A (1mg per mouse). The results are shown in Figure 6b and demonstrate that the microencapsulated cells plus ponasterone A treatment completely inhibited the growth of SK-OV-3 cells. SK-OV-3 cells showed considerable growth in the absence of ponasterone A.